Eurycomanone Exerts Antiproliferative Activity via Apoptosis in HeLa Cells

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Abstract

Eurycomanone is a cytotoxic bioactive ingredient found in Eurycoma longifolia *Jack. It demonstrated a general cytotoxic response against many epithelial cell types. The present study investigated the antiproliferative activity of eurycomanone. It showed antiproliferative effects on cancerous cell lines (Caov-3, HeLa, Hep G2, HM3KO and MCF-7) and relatively nontoxic on noncancerous cell lines (MDBK, Vero). The mode of cell death in HeLa cells induced by eurycomanone was determined by TUNEL assay. It detected the fluorescence in the nuclear region thereby indicating the presence of DNA fragmentation. The fluorescence became more intense with longer treatment durations. The apoptotic morphology of HeLa cells induced by eurycomanone was further confirmed by staining with nuclear fluorochrome Hoecsht 33258. Similar morphology was shown by HeLa cells treated with tamoxifen which acted as positive control. In short, eurycomanone proves to be cytotoxic towards HeLa cells by triggering apoptotic cell death.*

Keywords : *eurycomanone, HeLa cells, apoptosis*

I. Introduction

Eurycoma longifolia Jack is one of the popular folks medicines of South East Asia including Myanmar, Indochina, Thailand, Laos, Cambodia and Malaysia (Kuo et al. 2004). *E.longifolia* is identified locally as Tongkat Ali in Malaysia, Pasakbumi or Bidara Pahit in Indonesia, Ian-don in Thailand and Cay ba binh translated as a tree which cures hundreds of deseases (Kardono et al, 1991).

Several classes of compounds have been isolated and identified, including quassinoids (Morita et al, 1993; Darise et al. 1982; Ang 2002), canthin-6-one alkaloids (Kardono et al. 1991; Mitsunaga et al. 1994), β-carboline alkaloids (Kardono et al 1991; Kuo et al. 2004) and squalene derivatives (Morita et al. 1993).

Previous studies on the methanolic extract from the root of *E.longifolia* showed the antiproliferative activity towards a panel of cancer cell lines comprising a number of human cancer cell types (breast, colon, fibrosarcoma, lung, melanoma, KB and P-388 murine lymphocytic leukemia (Kardono et al. 1991). Proceed study of methanolic extract showed induction of apoptosis on human breast cancer cell lines (MCF-7) via decreasing of Bcl-2 proto-oncogene (Tee and Azimahtol, 2005).

Apoptosis is the ability of a cell to self-destruct by the activation of an intrinsic cellular suicide program when the cells are no longer needed or when they are seriously damaged (Huang et al 2003). Apoptosis is an active form of physiological cell death.. which is essential for development and for cellular and tissue homeostasis (Bohm and Schild, 2003). In cancer therapy, one approach that suppress the tumour growth is by activating the apoptotic machinery in the cell (Lowe and Lin, 2000). Moreover, the apoptotic process includes a mechanism that organized both the packaging and disposal of dead cells, thereby preventing inflammation of the surrounding tissue (Zornig et al., 2001). Evidence obtained during recent years is beginning to establish that a large of cancer chemotherapy agents affect tumor cell killing *in vivo* and *in vitro* through launching the apoptosis cascade (Markin and Dive, 2001). The apoptotic cell is characterized by loss of cell volume, plasma-membrane blebbing, nuclear condensation, chromatin aggregation and endonucleocytic degradation of DNA into nucleosomal fragments (Bohm and Schild, 2003).

The objective of this study is to evaluate the antiproliferative effect of eurycomanone and the ability of eurycomanone to induce apoptotic cell death in human cervical carcinoma (HeLa) cells.

II. Materials And Methods

Plant materials, *E.longifolia* roots were provided by Prof Dr. Azimahtol Hawariah Lope Pihie (National University of Malaysia).

Compound extraction, Eurycomanone extracted from the root of E.longifolia as previously described (Darise et al. 1986). Root of the plant were dried and ground extracted with MeOH. The MeOH extract was concentrated to dryness. A suspension of the residue in H_2O was extracted with Et_2O and then with n-BuOH saturated with H_2O. The BuOH layer was evaporated to dryness and the residue was chromatographed on a Silica gel column, elution with EtOAc-EtOH-H_2O (100:10:1).

Cell culture conditions, Hela, CaOv-3, HepG2, MCF-7, HM3KO, Vero, MDBK were obtained from American type culture collection (ATCC) and maintained in DMEM that was supplemented with 5% fetal bovine serum and penicillin streptomycin.

Cell proliferation assay, The antiproliferative effect was evaluated by obtaining the IC_{50} values for these cell lines as previously described (Lin and Hwang, 1991; Lee and Azimahtol, 2003). The cells were plated onto each well in a volume of 0.1 ml and were allowed to attach overnight at 37 ^oC and then the seeding medium was removed and replaced with fresh medium containing varying concentration of compound. The cells were maintained for 3 days and the antiproliferative activity was determined using methylene blue staining. Glutaraldehyde was added to each well to final concentration of 2.5 % (v/v) and fixed for 15 minutes. After washing with 0.15 M NaCl the fixed cells were stained with 0.1 ml of 0.05% methylene blue solution for 15 minutes. The excess dye elution was washed out and 0.33 M HCl (0.2 ml per well) was added. The absorbance read at 660 nm.

TUNEL assay, DNA fragmentation, that is characteristic of apoptotic cells, was examined by Tdt-mediated dUTP nick end labelling (TUNEL) with the apoptotic detection kit, Florescein (Promega) according to the manufacturer's instruction. Tamoxifen used as positive control.

Nuclear staining assay. Staining with Hoechst 33258 was performed as previously described (Hishikawa et al. 1999). The cells were grown in poly l-lysin slide, remove the old medium and replaced with fresh medium containing the compound. After incubation cells were washed with PBS and then fixed with 4% paraformaldehyde for 30 minutes. After washing, the cells were incubated with Hoechst 33258 (Sigma) at a final concentration of 30 μg/ml. Nuclear morphology were then determined with *Leica* fluorescent microscope

III. Results

Eurycomanone significantly reduced cell viability (Caov-3, Hela, HepG2, HM3KO, MCF-7) in a dose dependent manner. It is relatively nontoxic to normal cells (MDBK, Vero) (Fig 1). The IC_{50} of eurycomanone on cancerous cell lines are less than 20 μg/ml. These finding suggested its potential as antiproliferative agent since the IC_{50} is less than 20 μg/ml (Wall et al. 1987).

The mode of killing that is induced by most anticancer agents is by apoptotic cell death. The antiproliferative activity shown by eurycomanone could possibly due to the induction of apoptosis. We used the TUNEL and nuclear staining with Hoechst 33258 to prove this hypothesis.

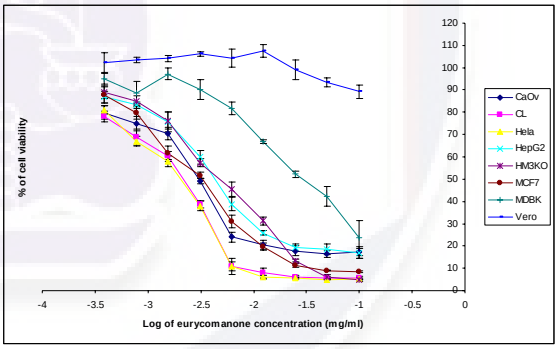

Fig 1. Effect of eurycomanone on cell viability

Table 1. IC_{50} values of eurycomanone and positive control (tamoxifen) towards cancerous and noncancerous cell lines

Cell lines	IC_{50} (μg/ml)	
	eurycomanone	Tamoxifen
Caov-3	3.03±0.14	1.92±0.04
Hela	2.13±0.09	8.13±0.19
HepG2	4.02±0.12	2.16±0.08
HM3KO	4.21±0.2	
MCF-7	3.63±0.11	
MDBK	28.94±1.7	10±0.13
Vero	>>>	

DNA fragmentation that is characteristic of apoptotic cells could be determined by Tdt-mediated dUTP nick end labelling (TUNEL). DNA fragmentation generates 3'-OH DNA ends, which can be labeled with fluorescein-12-dUTP using the enzyme Terminal Deoxynucleotidyl Tranferase (TdT). The fluorescein-12-dUTP-labeled DNA could be visualized by fluorescence microscope. Here, we labeled the eurycomanone-treated cells to visualize the extent of DNA fragmentation in a time-course dependent manner (Fig 2).

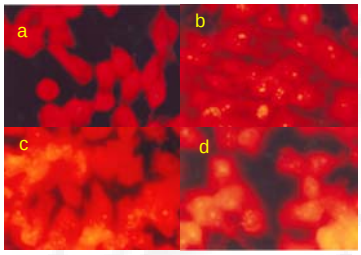

Fig 2. TUNEL labeling of HeLa cells. (a) untreated cells. No flurescence detected in the nucleus, as the cells were not apoptotic and did not exhibit DNA fragmentation. Eurycomanone treated cells at 24 h (b), 48 h (c) and 72 h (d) showed fluorescence in the nuclear region, indicating apoptosis. The fluorescence became more intense with longer treatment duration.

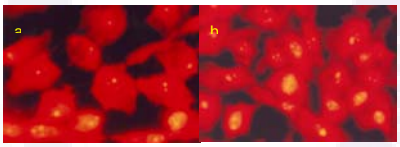

Fig 3. TUNEL labeling of tamoxifen-treated HeLa cells at 24 h (a), 72 h (b)

The apoptotic morphology of HeLa cells induced by eurycomanone was further confirmed by staining with nuclear fluorochrome Hoecsht 33258.

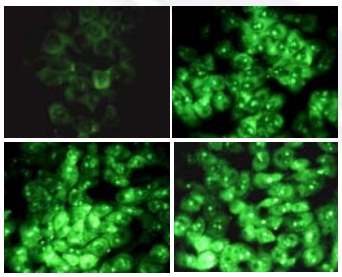

Fig 4. Nuclear staining of HeLa cells with Hoechst 33258. Cells were treated with eurycomanone compare to untreated controls. Untreated cells (a) eurycomanone treated cells at 24h (b), 48h (c), 72h (c). There are no fluorescence detected from untreated cells, while the eurycomanone treated cells showed fluorescence of apoptotic bodies.

IV. Discussions

Plants are a valuable source of new natural products. Despite the availability of different approaches for the discovery of therapeuticals, natural products still remain one of the best reservoirs of new molecules (Pezzuto 1997). Natural plant products play an important role in chemotherapy, having contributed considerably to approximately 60 available cancer chemotherapeutic drugs (Kinghorn et al. 1999). The need to develop more effective antitumor drugs has prompted investigators to explore new sources of pharmacologically-active compounds, especially from natural products.

In cancer therapy, one approach that suppress the tumour growth is by activating the apoptotic machinery in the cell (Lowe and Lin 2000). There are three ways for studying cell death that use DNA stains: dye exclusion method, morphological changes and profile of DNA content. In the study of morphological changes, certain dye or fluorochrome can be used to view the structure of viable cells and apoptotic cells.

Morphological changes of apoptotic cells, chromatin condensation and formation of apoptotic bodies could be determined using Hoecsht 33258 staining. It will bind specifically to double stranded DNA and emit green fluorescence. . Hoecsht 33258 will stain both of healthy and apoptotic cells, but apoptotic cells will stain strongly distinguished from healthy that is homogenously.

Untreated HeLa cells stain homogenously with Hoecsht 33258, no fluorescence detected indicating the cells are healthy while the eurycomanone-treated cells showed of intense fluorescence indicating that cells were apoptotic.

DNA fragmentation that is characteristic of apoptotic cells could be determined by Tdt-mediated dUTP nick end labelling (TUNEL). DNA fragmentation generates 3'-OH DNA ends, which can be labeled with fluorescein-12-dUTP using the Terminal Deoxynucleotidyl Tranferase (TdT) enzyme . The fluorescein-12-dUTP-labeled DNA could be visualized by fluorescence microscope.

The healthy, untreated HeLa cells did not emit yellow fluorescence indicating that none of the cells underwent apoptosis thus dUTP was not being incorporated into the DNA of the cells. Red stain were caused by Propidium iodide. When the cells fixed with 4% paraformaldehyde, it made the cell membrane permeable to propidium iodide. However, this did not indicate that cells were apoptotic.

The eurycomanone-treated HeLa cells showed yellow fluorescence with TUNEL assay indicating the DNA fragmentation. After 24 h treatment a few fluorescent detected and the fluorescense became more intense with longer treatment duration. The similar morphology showed by tamoxifen-treated HeLa cells.

V. References

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